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PRINCIPAL INVESTIGATOR: Yunge Zhao, Ph.D.

CONTRACTING ORGANIZATION: Florida State University
Tallahassee, FL 32306

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14. **ABSTRACT**
Cells grow in three-dimensional (3D) environments under normal physiological and pathological conditions, while most in vitroexperiments are performed utilizing monolayer cell cultures. Evidence suggests that gene and protein expression patterns are distinctly different in 3D cultures when compared with the monolayer (2D) cultures commonly employed. We have therefore created a novel polyvinyl alcohol (PVA) 3D cell culture system to study human breast cancer cell progression and cancer cell-stromal cell interactions. Results have demonstrated that human breast cancer cell (MDA-MB-231) and human fibroblast cell (HFL-1) attachment is varied in the presence of different extracellular matrix components, and when MDA-MB-231 cells invade into the bottom layer of this PVA 3D culture system, containing HFL-1 cells, breast cancer tissue-like structures are formed. The MDA-MB-231 cells have been identified by immunohistochemistry in conjunction with an appropriate epithelial cell marker. However, the attachment of MDA-MB-231 cells onto the PVA sponge itself is not satisfied, necessitating the switching to use the Gelfoam, a porous gelatin block, for the proposed system. Changing the original scheme to use Gelfoam hasyielded several interesting and useful findings. MMP-26 knockdown by siRNA was accomplished in MDA-MB-231 cells at 90%, facilitating further investigation into the roles of MMP-26 in breast cancer cell invasion utilizing this 3D cell culture system.

15. SUBJECT TERMS
3-dimensional cell culture system, matrix metalloproteinase-26, cancer cell-stromal cell interactions, cancer cell invasion

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Introduction

Proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM) is a critical prerequisite for breast carcinoma cell invasion, and matrix metalloproteinases (MMPs) are thought to play important roles during this process. MMP-26 (endometase/matrilysin-2), a novel member of this enzyme family recently cloned and characterized [1], is primarily expressed in cancers of epithelial origin, such as those of the breast, endometrium, and prostate. We have shown that MMP-26 promotes prostate cancer cell invasion by activating another powerful MMP, pro-gelatinase B (MMP-9) [2]. We have also demonstrated that the MMP-26 protein is highly expressed in human breast ductal carcinoma *in situ* (DCIS) and during the early stages of human breast ductal invasive carcinoma [3], while little to no MMP-26 protein expression is detected in normal and hyperplastic tissues. MMP-26 mRNA has also been detected in the human breast carcinoma cell line MDA-MB-231, but as the expression patterns of genes [4] and proteins [5] are distinctly different in three-dimensional (3D) cultures when compared with the monolayer (2D) cultures commonly employed, this proposal seeks to create a working 3D cell culture system incorporating "stromal" HFL-1 fibroblast cells for the evaluation of carcinoma invasion in an environment that more closely mimics that found under physiological conditions. The effects of function-blocking antibodies and siRNA knockdown of MMP-26 expression in MDA-MB-231 cells have been evaluated in 2D and 3D systems for comparative analyses of breast cancer cell invasion during carcinoma progression.

Body

This purpose of this study was to determine the effects of blocking matrix metalloproteinase (MMP-26) activity and expression in a three-dimensional (3D) cell culture system utilizing function-blocking MMP-26 antibodies and a knockdown of MMP-26 protein expression. The effects upon cancer cell invasion were to be evaluated and compared in conventional two-dimensional (2D) systems and the novel 3D culture system utilizing the breast cancer cell line MDA-MB-231. The 3D system initially proposed utilized layers of polyvinyl alcohol (PVA) sponges seeded with human breast cancer (MDA-MB-231) cells on the top layer and human fibroblast (HFL-1) cells on the bottom layer in an attempt to better mimic actual *in vivo* environments. As the proposed system ultimately proved intractable, *Gelfoam*, a porous gelatin block, was later incorporated into these cell culture and cell invasion systems, yielding results that were superior to those obtained with the PVA sponge system alone.

Task 1: To establish the three-dimensional polyvinyl (PVA) sponge culture system and to obtain the optimum culture conditions for breast cancer cell line MDA-MB-231 and fibroblast cells.

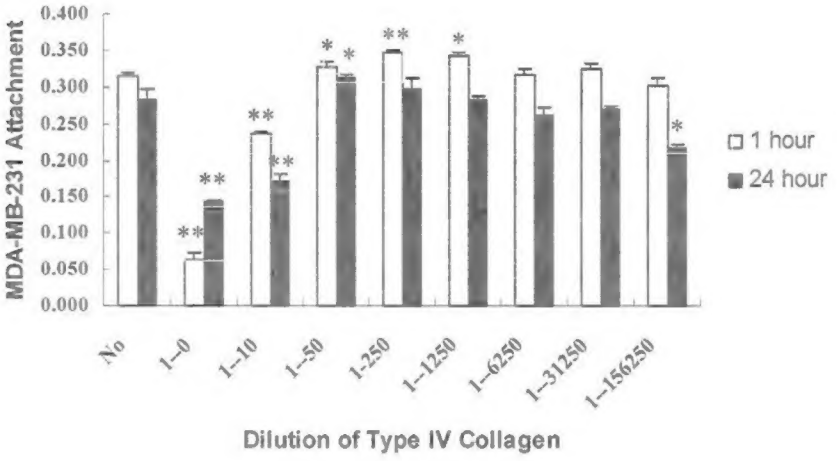
Task 1 (a & b): Establish the three-dimensional PVA sponge culture system and work out the optimum conditions for MDA-MB-231 in the top layer and for the human fibroblast cells in the bottom layer.

MDA-MB-231 and HFL-1 Cell Attachment Assays

Growth of the MDA-MB-231 and HFL-1 cell lines in the proposed PVA sponge culture system was attempted, however, it was determined that the cells did not attach to the PVA sponge in an acceptable fashion. This was followed by an attempt to identify which ECM components could enhance cell attachment to the PVA sponge, and the optimum concentration for these components, as cell attachment to ECM constituents is fundamental to the processes of cancer cell invasion and metastasis. Attachment assays incorporating MDA-MB-231 and HFL-1 cells were performed utilizing 5 different ECM components, including *Matrigel* (BD Bioscience, Bedford, MA), fibronectin (Gibco Invitrogen Corporation), type IV collagen (Sigma St. Louis, MO), type I collagen (Sigma St. Louis, MO), and laminin (Trevigen). For these assays, 96-well plates were coated with 80 µl of the different ECM components at varying concentrations prior to overnight drying in a laminar flow cell culture hood in the presence of ultraviolet (UV) decontamination. Cells were then cultured at a concentration of 1.0×10^4 /well in Dulbecco's Modified Eagle's Medium (DMEM) (LTI) (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin G and 100 µg/ml streptomycin at 37°C in 5% CO_2 for 1 to 24 hours. Cultured cells were then washed three times with PBS prior to staining with 0.1% Crystal Violet for 2 minutes. After the staining solution was removed, absorbance values were read at 492 nm using an Automatic Microplate

Reader (Titertek Multiskan MC-340, Flow Laboratories, McLean, Virginia). These experiments were repeated three times, with each iteration yielding similar results, as detailed in **Figure 1.**

A



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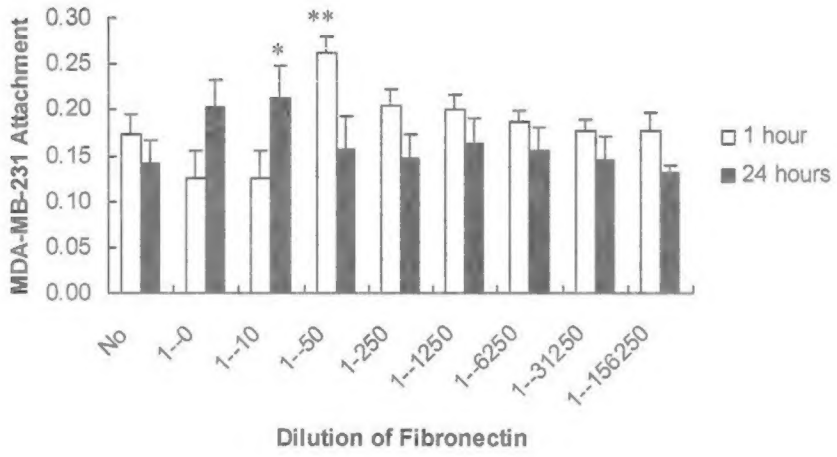

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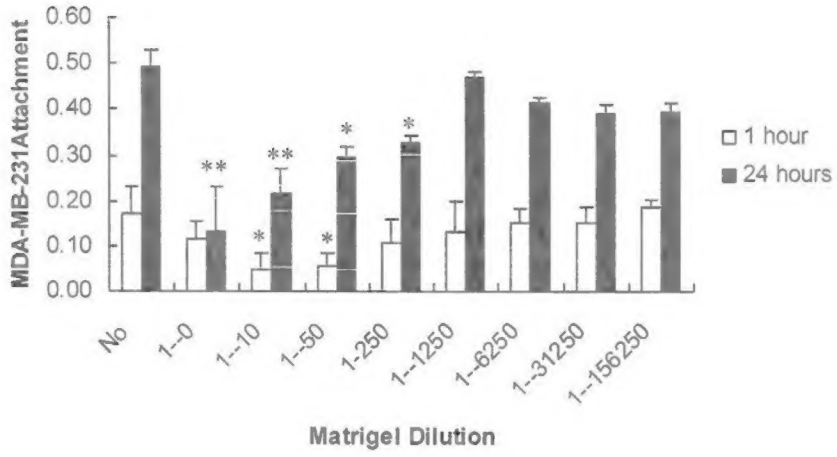

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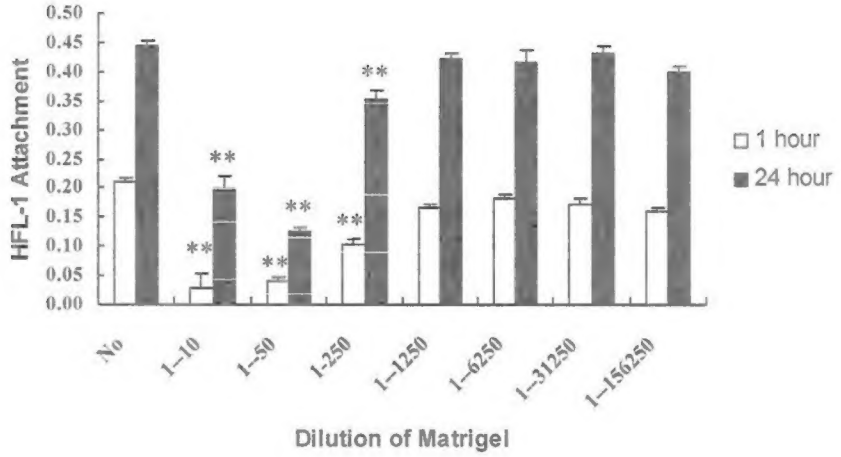
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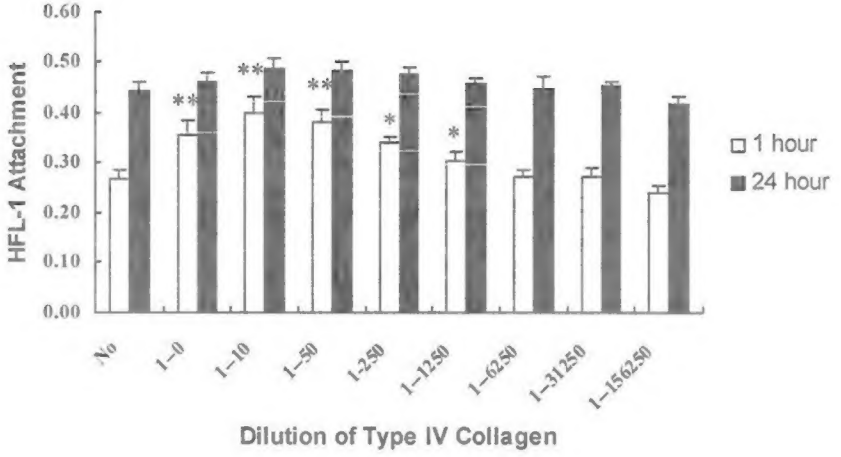
1–156250
Dilution of Matrigel

E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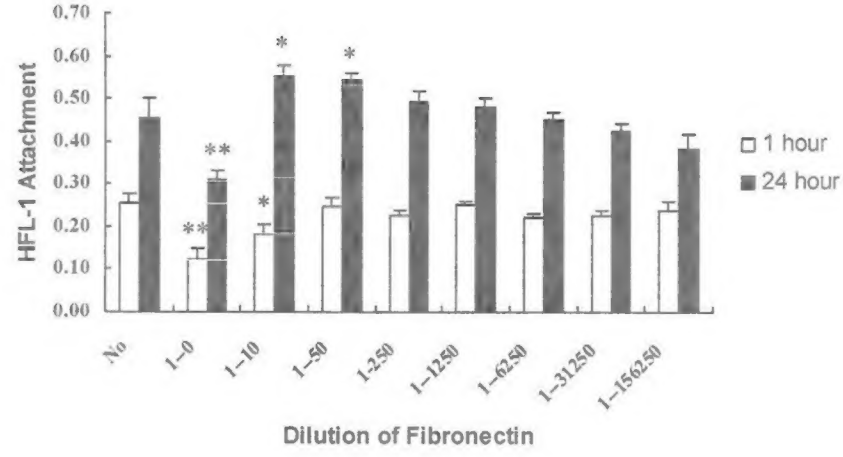
Dilution of Type IV Collagen

F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1 hour
24 hour
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1–10
1–50
1-250
1–1250
1–6250
1–31250
1–156250
Dilution of Fibronectin

Figure 1. MDA-MB-231 cell and HFL-1 cell attachment on different ECM components. **A-C:** MDA-MB-231 cell attachment on Type IV collagen, fibronectin and *Matrigel*, respectively. **D-F:** HFL-1 cell attachment on *Matrigel*, type IV collagen and fibronectin, respectively. The original concentration for type IV collagen and fibronectin was 1 mg/ml, and for *Matrigel*, 13.9 mg/ml (56% laminin, 31% type IV collagen, and 8% entactin). Attachment values were derived from OD_{490} values, which were subsequently used for comparative statistical analyses by analysis of variance (ANOVA). Statistical analysis of all samples was performed with the least significant difference (LSD) correction of ANOVA for multiple comparisons. Data represent the mean ± standard deviation (SD) from three experiments, where differences with $P < 0.05$ were considered to be significant. **: $P < 0.01$; * $P < 0.05$.

The results of the attachment assays revealed that type IV collagen at a 1:250 dilution enhanced the attachment of both MDA-MB-231 and HFL-1 cells (**Figure 1A** and **1E**), while *Matrigel* coatings diminished the attachment of both cell lines (**Figure 1C** and **1D**). Therefore, type IV collagen was used to coat the PVA sponge rather than *Matrigel*, which been originally proposed.

Identification of MDA-MB-231 Cells in a HFL-1 / MDA-MB-231 Co-culture System

HFL-1 cells were seeded at 2.5×10^4 /well in a 24-well plate (or 100 mm^2 dish) and were cultured for 24 hours, after which time 2.5×10^4 MDA-MB-231 cells/well were added and growth of the culture was continued for an additional 24 hours. The co-cultured cells were fixed with 4% paraformaldehyde (PFA, Sigma) solution, and were then stained by immunocytochemistry according to our previous reports [2, 3]. The primary antibody was a monoclonal mouse anti-human epithelial membrane antigen (EMA) IgG (DAKO Corporation, Carpinteria, CA). The secondary antibody was an alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (Sigma Immuno Chemicals, St. Louis, MO). SIGMA FAST™ Fast Red TR/Naphthol AS-MX (Sigma) was used as substrate to detect positive signals, as shown in **Figure 2**.

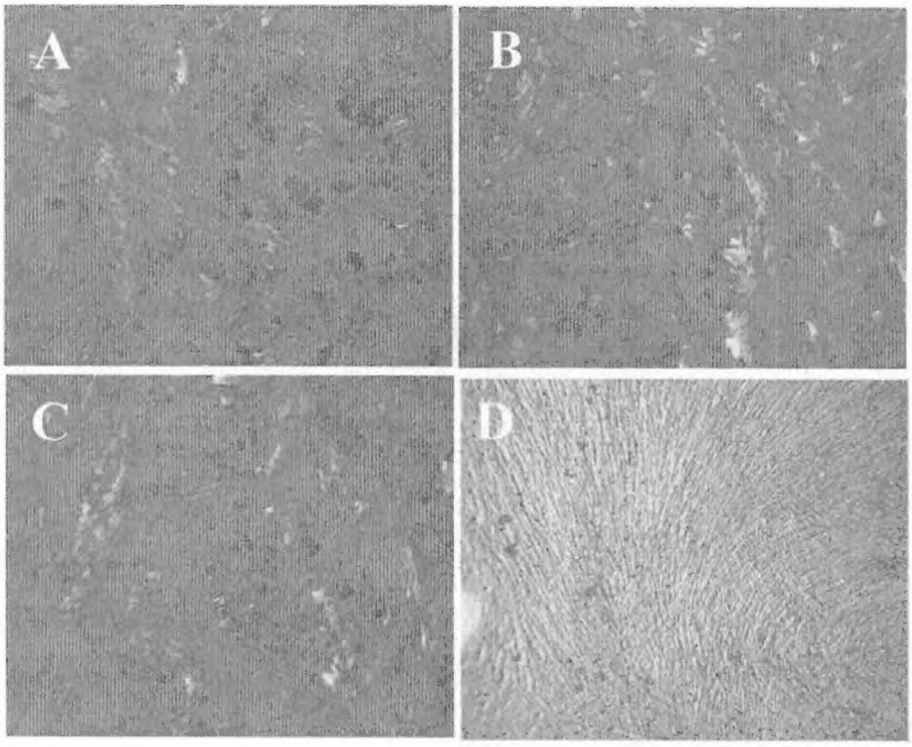


Figure 2. Immunohistochemical staining of epithelial membrane antigen (EMA) in MDA-MB-231 / HFL-1 cell co-culture systems. In this co-culture system, an anti-EMA antibody could be used as a marker to differentiate MDA-MB-231 cells from HFL-1 cells (Figure 2A-C), as the HFL-1 cells demonstrated only background staining signals (Figure 2D). Under normal culture conditions, HFL-1 cells grow evenly, in a 2-dimensional fashion; but when co-cultured with MDA-MB-231 cells, the HFL-1 cells formed 3D-structures, and the MDA-MB-231 cells clustered as pathological tissues.

Growth of MDA-MB-231 and HFL-1 cells in PVA sponge

A great deal of time and energy was devoted to this particular task. Many methods were used to pre-treat the PVA sponge prior to cell seeding, including autoclaving and immersion in 70% ethanol, but both of these methods left the sponge shrunken and deformed. Overnight exposure to UV light resulted in decontamination of only the sponge surface given the thickness of the sponge itself. Finally, the sponge was cut into two parts, soaked in PBS solution with 1% penicillin and streptomycin under UV overnight, then dried in a laminar flow hood under UV. Following pre-treatment of the PVA sponge, the sponge was coated with 100 µl type IV collagen (1:250 dilution) and allowed to dry under UV light in a laminar flow hood. The sponge was then incubated in fresh media for 30 minutes prior to seeding with MDA-MB-231 and HFL-1 cells. Seeding was accomplished by incubating 80 µl MDA-MB-231 or HFL-1 cells ($2.5X10^5$ cells) with a sponge in the autoclaved cap of a 1.5 ml Eppendorf tube. The

caps and sponges were then placed into covered 24-well plates and incubated at 37 ° C in 5% CO_2 for 1 hour without adding culture media. The sponge was then moved to a new 24-well plate in the presence of fresh media. The sponge was incubated for 5 days, with replacement of media every two days.

Construction of the 3-dimensional (3-D) cell culture system

To separate the top and bottom layers we utilized a special insert that is slightly raised within the well, supported by three stands, and containing a polycarbonate membrane with 8 µm pores at its base. In this way, the bottom layer of sponge remained in contact with the ECM/BM layer while the upper chamber was seeded with MDA-MB-231 cells. After 48 hours invasion, the bottom layer sponge was separated from the insert and growth was continued for an additional 3 days. The sponge was then washed with PBS and embedded in Optical Cutting Temperature compound prior to its incubation at -80 °C for sectioning into 50 µm sections and mounting onto slides. Standard immunohistochemical staining was then performed according to our previous reports [2, 3], with representative results shown in **Figure 3**. The primary antibody was a monoclonal mouse anti-human epithelial membrane antigen (EMA) IgG (DAKO Corporation, Carpinteria, CA).

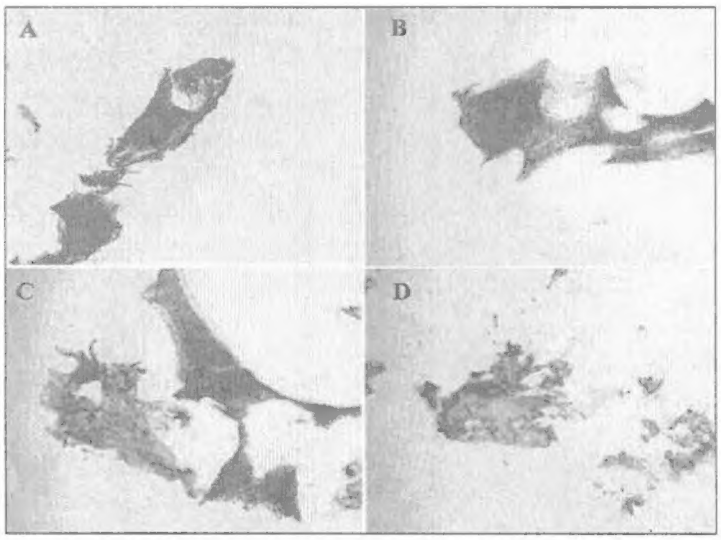


Figure 3. Immunostaining of epithelial membrane antigen (EMA) molecules in the 3D culture system. In the 3-D culture system, MDA-MB-231 cells were stained red, indicating the presence of EMA, while the underlying HFL-1 cells had only weak staining signals (Figures 3A, C, and D). A normal mouse IgG control is shown in Figure 3B. In this 3D-culture system, the MDA-MB-231 cells formed cancer cell clusters similar to cancerous breast tissues (Figures 3C and D). Therefore, this model may represent a useful tool to study epithelial-stromal cell interactions and cancer cell invasion in a 3D environment that mimics physiological conditions.

Difficulties and Solutions for Task 1 (a & b):

(1) The MDA-MB-231 and HFL-1 cells did not attach well to the PVA sponge, even when precoated with type IV collagen, which was shown to facilitate binding of these cells.

Solution: *Gelfoam* was utilized in addition to PVA, and *Gelfoam* was found to promote attachment in a manner superior to that of even the coated PVA sponge.

(2) Cells were not evenly distributed within the PVA sponge, and therefore, the number of invaded cells may not be indicative of invasion through a true 3D environment.

Solution: The sponge was cut into smaller sizes ($5\times4\times3$ mm^3), cells were suspended at higher concentrations (1.25×10^8/sponge block), and care was taken to ensure that the cells were seeded in the center of the sponge. Floatation of the sponge was also deterred by the weight of the media in the upper chamber.

(3) It is very difficult to cut PVA sponge to a 10 μm thickness.

A. As the media contained within the PVA sponge could not be completely evacuated prior its being embedded in Optical Cutting Temperature compound, the sponge was broken very easily. Evacuation of all media by squeezing the sponge was not considered, as this would most certainly effect the shape of the cells and their position within the sponge.
B. The average pore size of the PVA sponge was too large to accommodate the preparation of 10 μm sections, resulting in sponge fragments as opposed to a full section. It was later determined that 50 μm was the minimum thickness that consistently yielded intact slides.
C. At a thickness of 50 μm, immunohistochemical staining frequently results in dissociation of the sponge from the slide, as shown in **Figure 3D**.

Solution: To solve the difficulties enumerated above, the use of *Gelfoam* was incorporated into the design of the PVA sponge 3D culture system originally proposed.

Task 1 (c): Perform MDA-MB-231 invasion assays in the 3D culture system.

For three-dimensional (3D) invasion assays, *Gelfoam* (Pfizer) was cut into 6 x 7 mm^2 (diameter x thickness) sections for purposes of growing MDA-MB 231 cells, and 6 x 3 mm^2 sections (diameter x thickness) for purposes of growing HFL-1 cells. The MDA-MB-231 cells (0.5×10^6 cells/100 µl) were grown in *Gelfoam* sections for 9 days at 37°C in 5% CO_2 with media changes every 2 days, and HFL-1 cells (0.25×10^6 cells/100 µl) were grown in *Gelfoam* sections for 3 days. *Gelfoam* sections containing MDA-MB-231 cells were washed with serum-free media then pre-incubated in serum-free media in the presence or absence of antibodies for 1 hour, and were then placed into the upper chambers of precoated Boyden inserts and incubated for 24 hours. The *Gelfoam* sections containing HFL-1 cells, or *Gelfoam* without cells (as a control), were placed beneath the inserts. Following the incubation, invading cells were stained with 0.1% crystal violet solution and photographed with a QImaging RETIGA EXi digital camera (Canada) under a Leica DMIRE2 microscope using IPLab software. The cells were then counted using Photoshop (Adobe) software. For statistical analyses, the number of invading cells treated with preimmune IgG was assumed to reflect 100% cell invasion. The ratio of the number of invaded cells treated with antibodies to untreated controls was used for subsequent comparative analyses by analysis of variance (ANOVA) utilizing the least significant difference correction. The HFL-1-*Gelfoam* sections that also contained invading MDA-MB-231 cells were frozen in Optical Cutting Temperature compound at -80°C for sectioning and immunostaining. The results of these analyses are summarized in the discussion of Task 2.

Task 1 (d): Evaluate the three-dimensional PVA sponge culture system by comparing with modified Boyden chamber (2D) invasion assays.

For two-dimensional (2D) invasion assays, Boyden chambers containing polycarbonate filters with 8-µm pores (Becton Dickinson, Boston, MA) were coated with 50 ul (0.25 mg/ml) type IV collagen (Sigma) in a laminar flow hood and were air-dried overnight with UV decontamination. Prior to cell seeding, 300 µl of serum-free media was placed into the inserts for 30 min incubation. Cell monolayers were first trypsinized, and were then washed twice with serum-free media. The cells were then resuspended in serum-free media and a cell count was obtained by hemocytometer. The 300 µl of blank rehydration media was then removed from the inserts and was replaced with 300 µl of the prepared cell suspension at a concentration of 1×10^6 cells/ml. This was followed by the addition of 500 µl of media containing 10% fetal bovine serum to the lower chambers. After 6-24 hours of incubation in a cell culture incubator

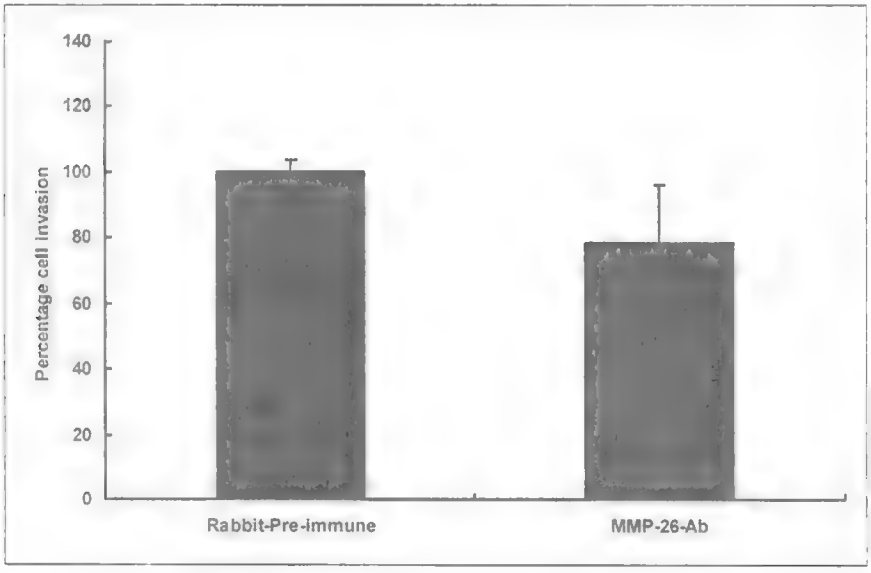
at 37°C in 5% CO_2, the media was removed and the invasive cells that had passed through the filters to the lower surface of the membrane were stained with a 0.1% crystal violet solution. Photographs of the membranes were obtained using a QImaging RETIGA EXi digital camera (Canada) under a Leica DMIRE2 microscope. The invading cells were counted using either Photoshop (Adobe) or Metamorph Systems software (Universal Imaging Corp.), and these numbers were utilized for subsequent comparative analyses by analysis of variance (ANOVA) as described above. The results of these analyses are summarized in the discussion of Task 2.

Task 2: **To investigate the role of matrix metalloproteinase-26 (MMP-26) in MDA-MB-231 invasion using the 3D culture system.**

Task 2 (a): Perform MDA-MB-231 invasion assays in the presence of purified anti-MMP-26 and/or anti-MMP-9 IgG and preimmune IgG.

Three-dimensional (3D) invasion assays were performed as described above in the presence or absence of specific and function-blocking antibodies targeting either MMP-26 or MMP-9 at a concentration of 25 µg/ml. The effects of MMP-26 inhibition in the 3D culture system were not of statistical significance, however, inhibition of MMP-9 resulted in a 40% decrease in the number of invading cells, as detailed in **Figure 4.** Two-dimensional (2D) invasion assays as described above were then performed for comparison, but in this case, neither the inhibition of MMP-26 or MMP-9 resulted in statistically significant decreases in invasion, as detailed in **Figure 5.**

A



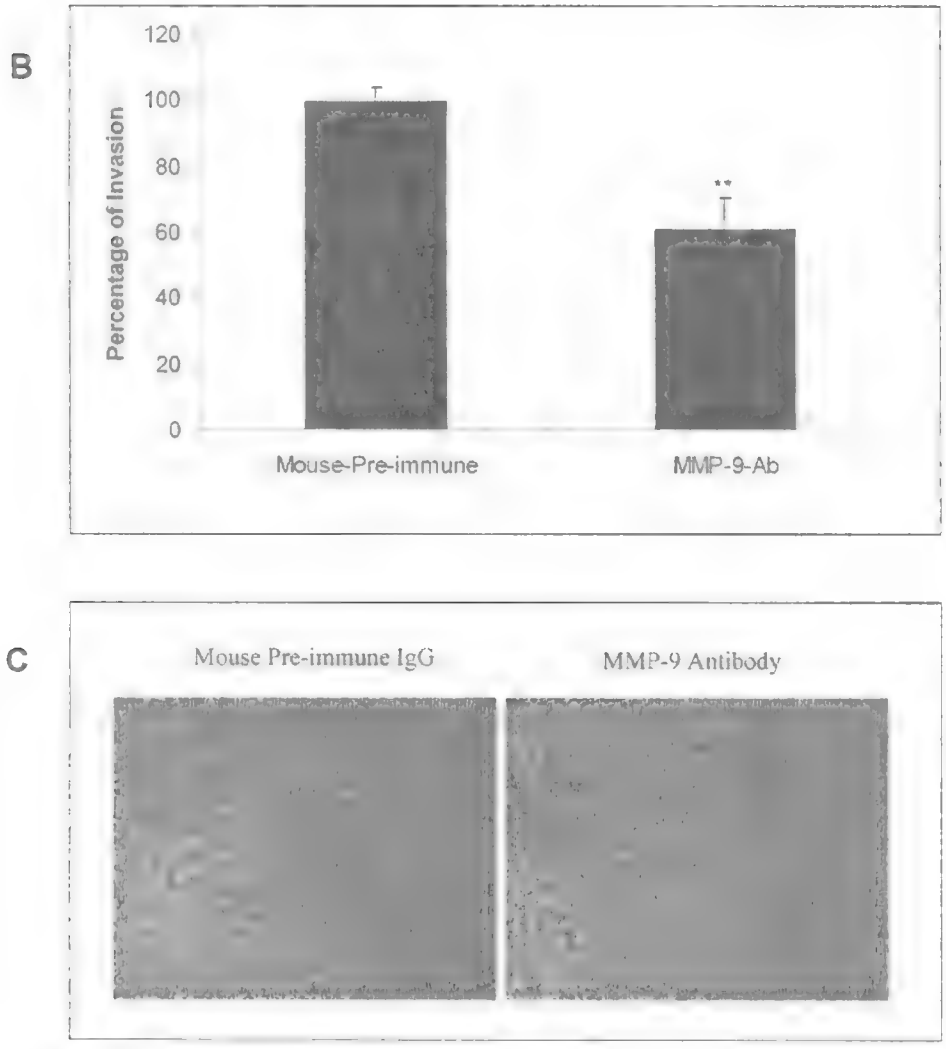
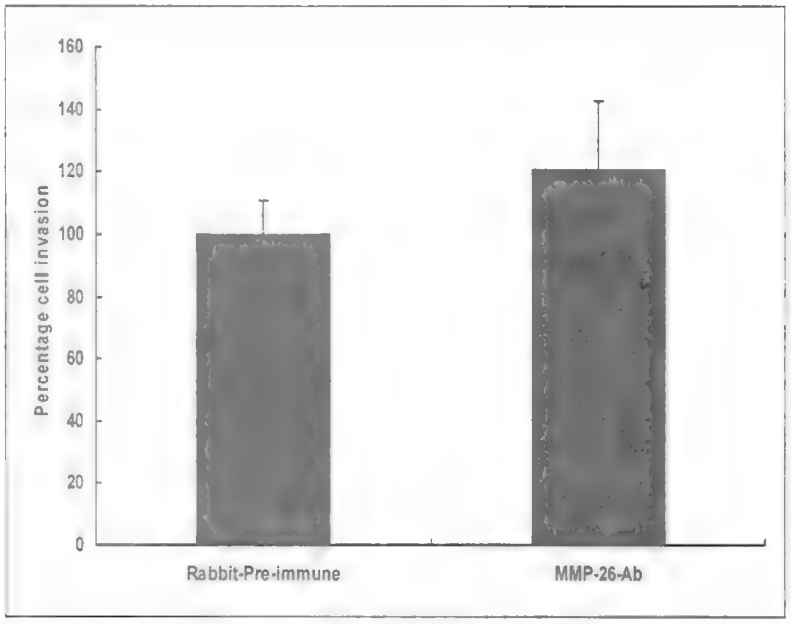


Figure 4. Effects of MMP-26 and MMP-9 antibodies on MDA-MB-231 cell invasion through type IV collagen in 3D cell culture systems. Invasion assays were performed with our modified Boyden chamber 3D culture system over the course of 24 hours as described in Methods. *A.* The effects of function-blocking anti-MMP-26 antibodies on MDA-MB231 cell invasion through type IV collagen. Normal rabbit IgG (preimmune IgG) was used as a control. All IgG concentrations were 25 µg/ml. *B.* The effects of function-blocking anti-MMP-9 antibodies on MDA-MB231 cell invasion through type IV collagen. Normal mouse IgG (preimmune IgG) was used as a control. All IgG concentrations were 25 µg/ml. *C.* Representative membranes illustrating cell invasion under differing conditions, photographed at 4X magnification. Data shown are the mean ± S.D. values from three separate experiments for each group. **, $p < 0.01$.



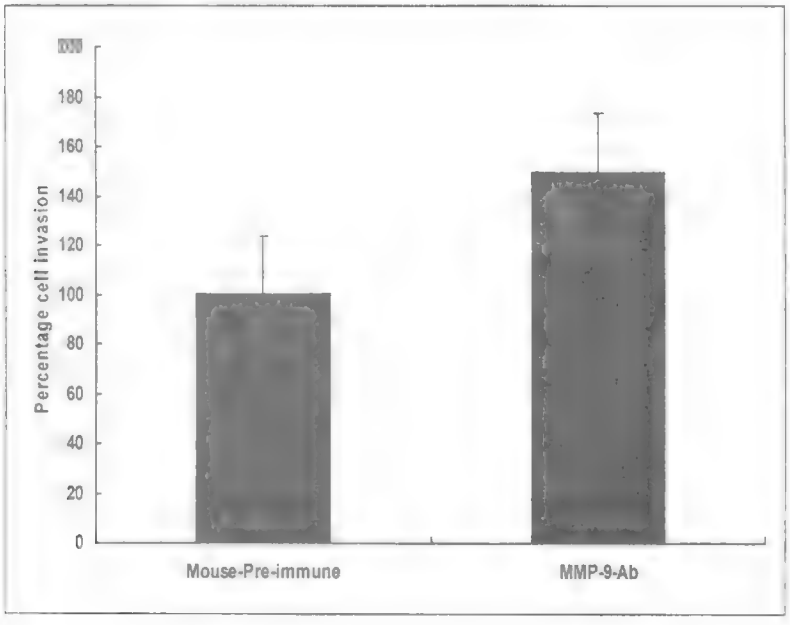


Figure 5. Effects of MMP-26 and MMP-9 antibodies on MDA-MB-231 cell invasion through type IV collagen in 2D cell culture systems. Invasion assays were performed with modified Boyden chambers over the course of 24 hours as previously described. *Top.* The effects of function-blocking anti-MMP-26 antibodies on MDA-MB-231 cell invasion through type IV collagen. Normal rabbit IgG (preimmune IgG) was used as a control. All IgG concentrations were 25 μg/ml. *Bottom.* The effects of function-blocking anti-MMP-9 antibodies on MDA-MB-231 cell invasion through type IV collagen. Normal mouse IgG (preimmune IgG) was used as a control. All IgG concentrations were 25 μg/ml. Data shown are the mean ± S.D. values from three separate experiments for each group. None of these findings displayed any significant difference.

Task 2 (b): Establish an MMP-26 antisense transfected stable MDA-MB-231 cell line.

The construction of the siRNA-expression plasmids was based on the siSTRIKE™ U6 Hairpin Cloning Systems (Promega). The vector includes a human U6 promoter, Amp^r / $Neomycin^r$ genes, and facilitates a sticky ends with downstream overhang PstI partial sites. The inserted hairpin sequence, which includes sense nucleotides, a loop-creating region, and anti-sense nucleotides, was designed using the siRNA Target Designer Program (www.promega.com/siRNADesigner/).

The sequences produced by this program are compared to all sequences in Genbank using the NCBI BLAST server. Among those sequences, only the specific MMP-26 target sequence was selected, and the control scramble sequence was designed in a similar fashion. The forward and backward sequences of the MMP-26 siRNA target insert and the scramble insert, respectively, are shown below:

5'-ACCGGAAGATGCAAGTGGAATAAAGTTCTCTTATTCCACTTGCATCTTCCTTTTTC -3'

5'-TGCAGAAAAAGGAAGATGCAAGTGGAATAAGAGAACTTTATTCCACTTGCATCTTC -3'

5'-ACCGATAGTGAACGGTAAGAAGAAGTTCTCTCTTCTTACCGTTCACTATCTTTTTC -3'

5'-TGCAGAAAAAGATAGTGAACGGTAAGAAGAGAGAACTTCTTCTTACCGTTCACTAT -3'.

After annealing, the DNA fragments were ligated, producing one new PstI site in addition to the existing PstI site, which were then used for selection.

Transfection of the MDA-MB-231 cells with MMP-26 siRNA target siSTRIKE™ resulted in an approximately 90% knock down of MMP-26 expression in this stably transfected cell line when compared to normal MDA-MB-231 cells and the scramble siRNA transfected cell line, as shown in **Figure 6**.

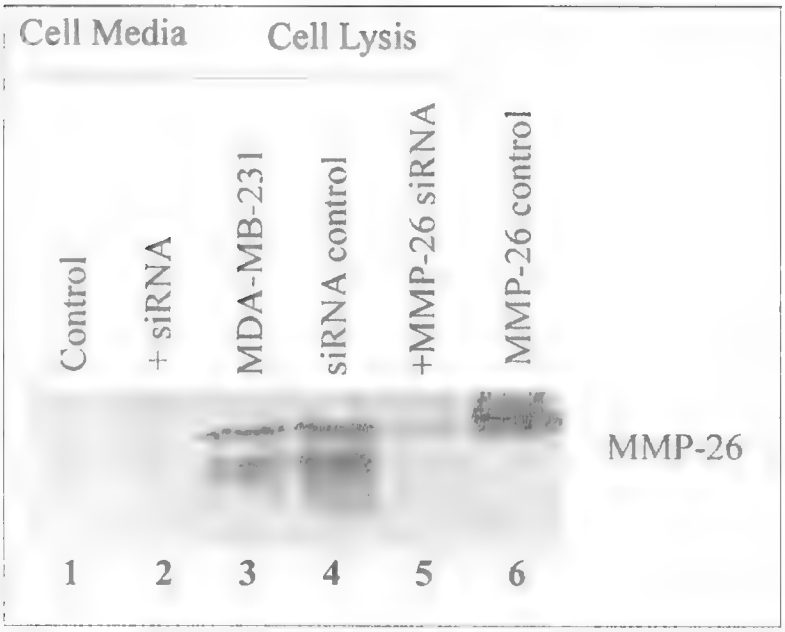


Figure 6. Expression of MMP-26 in normal and siRNA transfected MDA-MB-231 cells. The construction of the siRNA-expression plasmids was based on the siSTRIKE™ U6 Hairpin Cloning Systems (Promega). The vector includes a human U6 promoter, Amp^r / $Neomycin^r$ genes, and sticky ends with downstream overhang PstI partial sites. The inserted hairpin sequence, which includes sense nucleotides, a loop-creating region, and anti-sense nucleotides, was designed using the siRNA Target Designer Program. The sequences produced by this program are compared to all sequences in GenBank using the NCBI BLAST server. Among those sequences, only the specific MMP-26 target sequence was selected, and the control scramble sequence was designed in a similar fashion. Lanes 1 and 2 are cell culture media from normal MDA-MB-231 cells and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lanes 3 to 5 are cell lysates from parental MDA-MB-231 cells, MDA-MB-231 cells transfected with siRNA scramble inserts, and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lane 6 contains purified, recombinant MMP-26 expressed in BL21 (DE3)-competent *E. coli* cells. Lanes 3-6 were loaded with equivalent amounts of total protein.

Task 2 (c): Perform invasion assays using MMP-26 antisense transfected stable MDA-MB-231 cells in the 3D culture system.

Three-dimensional (3D) invasion assays incorporating transfected cells were performed as described above, and it was found that siRNA transfected cells invaded at a rate nearly double that of the non-silenced cells, as detailed in **Figure 7.** Two-dimensional (2D) invasion assays as described above were then performed for comparison, and in this case it was found that the siRNA transfected cells invaded at a rate nearly 2.5 that of the non-silenced cells, as detailed in **Figure 8.**

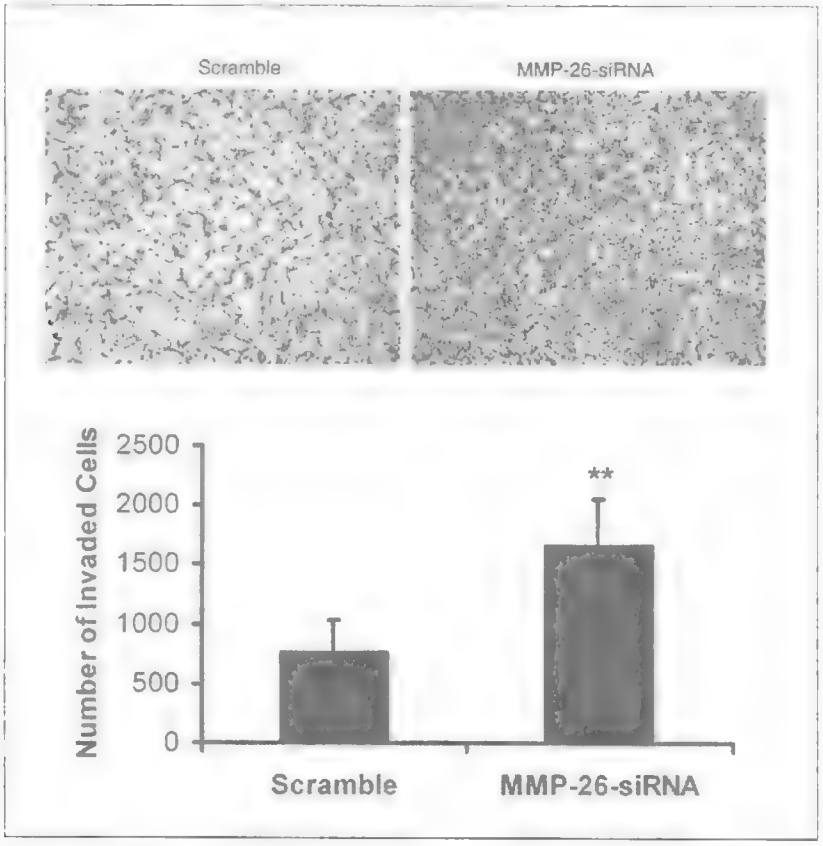


Figure 7. Promoting the invasion of MDA-MB-231 cells by MMP-26 siRNA transfection in 3D cell culture systems. The scramble and the MMP-26 siRNA transfected MDA-MB-231 cells were cultured in 500 µg/ml Geneticin (G418: Invitrogen) for two weeks, then grown in *Gelfoam* (0.5 X 10^6cells/100µl) for another 9 days. The MDA-MB-231-*Gelfoam* was then placed into the type IV collagen (0.25 mg/ml) pre-coated inserts and incubated for 24 hours at 37°C in 95% CO_2. The invading cells were stained with 0.1% crystal violet, and were then photographed and counted using Photoshop (Adobe) software. Data shown are the mean ± S.D. values from three separate experiments for each group. **, $p < 0.01$.

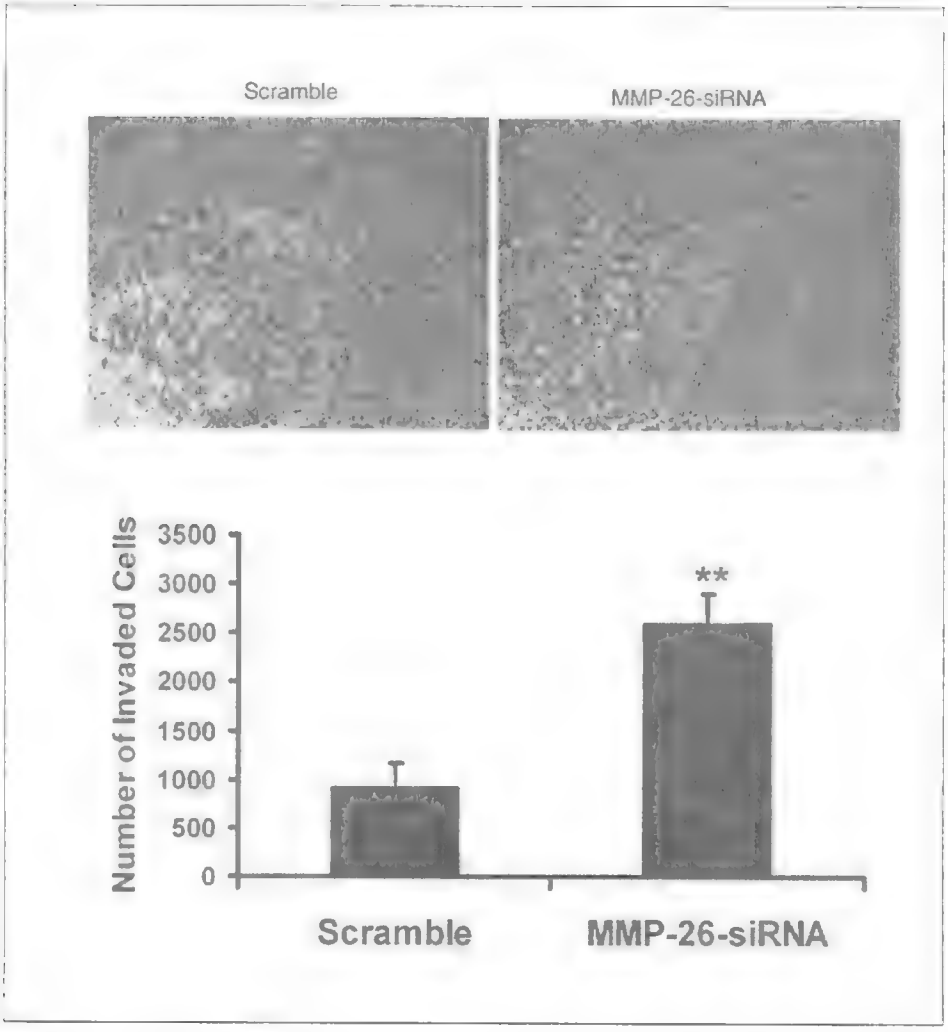


Figure 8. Promoting the invasion of MDA-MB-231 cells by MMP-26 siRNA transfection in modified Boyden chamber invasion assays. Modified Boyden chamber invasion assays were performed using scramble and MMP-26 siRNA transfected stable MDA-MB-231 cells. The scramble and the MMP-26 siRNA transfected MDA-MB-231 cells were cultured in 500 μg/ml Geneticin (G418; Invitrogen) for two weeks before seeding into the type IV collagen (0.25 mg/ml) pre-coated inserts, and were then incubated for 24 hours at 37°C in 5% CO_2. The invading cells were then stained with 0.1% crystal violet, photographed, and counted using Photoshop (Adobe) software. Data shown are the mean ± S.D. values from three separate experiments for each group. **, $p < 0.01$.

Key Research Accomplishments

1. Attachment assays revealed that type IV collagen (1 mg/ml) at a 1:250 dilution enhanced the attachment of both MDA-MB-231 and HFL-1 cells (**Figures 1A** and **1E**), while *Matrigel* coatings diminished the attachment of both cell lines (**Figures 1C** and **1D**). Therefore, type IV collagen was used to coat the PVA sponge rather than *Matrigel*, which been originally proposed.

2. In the 3D-culture system described in this report, the human breast cancer cell line MDA-MB-231 formed cancer cell clusters similar to that seen in cancerous breast tissues (**Figures 3C** and **3D**). Therefore, this model may represent a useful tool to study epithelial-stromal cell interactions and cancer cell invasion in a 3D environment designed to mimic that which occurs under normal physiological or pathological conditions.

3. Three-dimensional (3D) invasion assays revealed that inhibition of MMP-9 with specific, function-blocking antibodies resulted in a 40% decrease in the number of invading cells, as detailed in **Figure 4**.

4. Transfection of the MDA-MB-231 cells with MMP-26 siRNA resulted in an approximate 90% knock down of MMP-26 protein expression in this stably transfected cell line when compared to normal MDA-MB-231 cells and the scramble siRNA transfected cell line, as detailed in **Figure 6**.

5. Three-dimensional (3D) invasion assays incorporating MDA-MB-231 cells revealed that siRNA-transfected cells with diminished expression of MMP-26 invaded at a rate nearly double that of the non-silenced cells, as detailed in **Figure 7.**

6. Two-dimensional (2D) invasion assays incorporating MDA-MB-231 cells revealed that siRNA-transfected cells with diminished expression of MMP-26 invaded at a rate nearly 2.5 times that of the non-silenced cells, as detailed in **Figure 8**.

7. All the tasks listed in the original "Statement of Work" have been completed and *Gelfoam* has been used in stead of polyvinyl alcohol (PVD) for the 3-D culture system due to difficulties encountered with PVD.

Reportable Outcomes

1. The grant titled "*The Potential Role of Matrix Metalloproteinase-26 in Human Breast Carcinoma Invasion*" was submmited to The Susan G. Komen Breast Cancer Foundation on September 2, 2003. (Not funded).

2. Y. Zhao, A. Xiao, Y. Jin, M.A. Schwartz, and Q.-X. Sang (2004) Inhibition of MDA-MB-435 cell invasion by a synthetic matrix metalloproteinase inhibitor. The American Society for Cell Biology 44th Annual Meeting, December 4-8, 2004 Washington DC. Page 179, abstract # 2344, Poster # B351 (Attached in Appendices).

3. Y. Zhao, A. Xiao, and Q.-X. Sang (2005) A novel three dimensional cell culture system for human breast cancer: cancer invasion and cell-cell interactions. Era of Hope: *Proceedings of Department of Defense Breast Cancer Research Program Meeting* Poster P59-24, p417. June 8-11, 2005. Philadelphia, Pennsylvania. (Attached in Appendices)

4. Dr. Yunge Zhao has obtained a new research position at the Department of Pathology, University of Virginia.

5. Dr. Aizhen Xiao has obtained a new research position at the Department of Pathology, University of Virginia.

Conclusions

While it is unfortunate that a three-dimensional (3D) cell culture system comprised entirely of polyvinyl alcohol (PVA) sponge eventually proved to be intractable for a variety of reasons, switching the original scheme to use gelatin *Gelfoam* has yielded several interesting and useful findings.

During the optimization phase, it was shown that MDA-MB-231 and HFL-1 cells attach differentially to ECM components, and it was found that a coating of 1 mg/ml type IV collagen at a dilution of 1:250 provided the optimal attachment surface for these cell lines. Once a workable 3D system had been established, it was also shown that inhibition of MMP-9 with function-blocking antibodies reduced MDA-MB-231 cell invasion by 40%, while similar antibody treatment had no effect in conventional 2D invasion assay systems.

It was also shown that siRNA transfection could achieve a substantial knockdown of MMP-26 protein expression in MDA-MB-231 cells, with a diminution of nearly 90%, and the stable nature of this transfection facilitated their use in subsequent cell-based assay systems.

Invasion assays incorporating these transfected cells yielded surprising results in that these findings were contradictory to intuition. In 3D invasion systems, abrogation of MMP-26 expression resulted in a nearly two-fold increase in cell invasion, while in 2D invasion systems, the rate of cell invasion was nearly 2.5-fold that of controls. It is still unknown why targeting MMP-26 at the level of protein expression should yield results contrary from those obtained with function-blocking antibodies, but it does suggest roles for MMP-26 in cell invasion apart from the simple proteolysis of ECM and BM components. Ongoing research projects will attempt to address this paradox.

It was shown that this model may represent a useful tool for the study of epithelial cell-stromal cell interactions and cancer cell invasion in a 3D environment that mimics physiological conditions in a manner superior to that of conventional 2D monolayer systems.

All the tasks listed in the original "Statement of Work" have been completed and *Gelfoam* has been used in stead of polyvinyl alcohol (PVD) for the 3-D culture system due to difficulties encountered with PVD.

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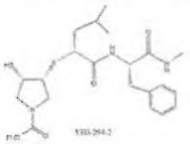
Inhibition of MDA-MB-435 cell invasion by a synthetic matrix metalloproteinase inhibitor

ABSTRACT

Matrix metalloproteinases (MMPs) have been implicated in the invasion and metastasis of human cancer cells by mediating the degradation of extracellular matrix components. We have demonstrated that MMP-26 promotes prostate cancer cell invasion through the activation of pro-MMP-9. To investigate the role MMP-26 in human breast cancer cell invasion, a novel MMP inhibitor was designed, synthesized, and tested. MDA-MB-435 breast cancer cells, expressing endogenous MMP-26 and MMP-9, were used in this cell culture study. Kinetic analyses revealed that a newly synthesized MMP inhibitor, YHJ-294, is selective and potent against MMP-26 as compared to MMP-7 and -12, which are highly homologous in MMP-26 according to protein sequence alignments. The IG values of YHJ-294-2 against MMP-26, MMP-7 and MMP-12 are 2.8 nM, 26 nM and 93 nM, respectively. YHJ-294-2 is stable in cell culture systems for at least 6 hours. YHJ-294-2 significantly inhibited the invasion of MDA-MB-435 cells through fibronectin and type IV collagen, while exerting no effects on cell attachment and proliferation after 24 hours. However, cell numbers were significantly reduced following consistent treatment with YHJ-294-2 for 7days. These results suggest MMP-26 involvement in human breast cancer cell invasion, and that YHJ-294-2 may become a drug candidate for the treatment of breast cancer.

Yeqin Dou, Aizhen Xiao, Yonghao Jin, Martin A. Schwartz, and Qing-Xiang Amy Sang
Department of Chemistry and Biochemistry,
Florida State University, Tallahassee, FL 32306-4390



YHJ-294-2

Fig 1. Structure of the synthetic mercaptosulfidyl inhibitor, YHJ-294-2

Inhibitor	*IG* (nM) MMP-26	MMP-7	MMP-12
YHJ-294-2	2.8	26	93
YHJ-294-1	650	3000	16000

Table 1. Inhibition of MMP-26 by new mercaptosulfidyl inhibitors.
Kinetic assays were performed using recombinant MMP-26, MMP-7 and MMP-12. The enzymes were pre-incubated with the new mercaptosulfidyl inhibitors for 30-60 minutes prior to the addition of the substrate, Mca-PLG[2pa]AR-NH2. The final concentrations were 0.5-7 nM for the MMPs and 1 uM for the substrate. The release of product was monitored by measuring fluorescence (excited at and emission wavelengths of 320nm and 393 nm, respectively) with a PerkinElmer luminescence spectrophotometer LS-50B.

Abbreviations: MMPs, matrix metalloproteinases; MMPI, MMP inhibitor.

*Supported in part by grants from Florida State University Research Foundation (to Q.-X.A.S. and T.-D.J.), the DOD Congressionally Directed Medical Research Programs (DAMD17-03-1-0402 (to Y.J.D.) and DAMD17-02-1-0258 (to Q.-X.A.S.).

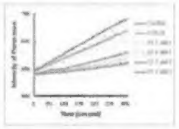


Fig. 2. Stability of YHJ-294-2 in MDA-MB-435 cell culture systems.
MDA-MB-435 cells were sub-cultured in DMEM medium with 10% fetal bovine serum for 18 hours. YHJ-294-2 was then added at varying concentrations and the conditioned media were collected after 6 hours for kinetic assays. The recombinant active metalloproteinase domain of MT1-MMP was used to detect the inhibitor activity in the conditioned media. The substrate utilized for these assays was M-1895. The final concentrations were 2.5 nM for MT1-MMP and 1μM for M-1895. The excitation wavelength was 328 nm, and the emission wavelength was 368 nm. The experiments were repeated three times, yielding similar results.

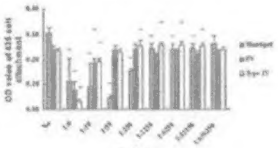


Fig. 3. Attachment of MDA-MB-435 cells on different extracellular matrix (ECM) components. After 96-well plates were pre-coated with ECM substrates, including Matrigel, fibronectin (FN), and type IV collagen (Type IV) at different concentrations, they were dried under a UV lamp. Identical cell counts (9.25X10⁴) were seeded into each well and incubated in 5% CO_2 at 37°C for 1 hour. The cells were stained with 0.1% crystal violet for 2 minutes and plates were read on a Thermo Multiscan MC-340 automatic microplate reader at 492 nm. Statistical analysis of all samples was performed with the least significant difference (LSD) correction of ANOVA for multiple comparisons. Data represent the mean ± standard deviation (SD) from three experiments where differences with $P<0.05$ were considered to be significant. ** $P<0.01$, * $P<0.05$

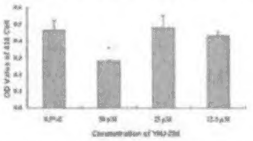
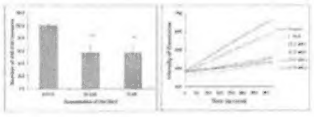


Fig. 4. Effects of YHJ-294-2 on MDA-MB-435 cell attachment on Type IV Collagen. Cell attachment assays were performed as described in Fig.3. These data represent the mean ± SD from three experiments. * $P<0.05$, 0.5%ΔΣ, 0.5% ethanol



A B

Fig. 5. Inhibition of invasion through Type IV collagen by YHJ-293-2.
(A) Inhibition of MDA-MB-435 cell invasion through type IV collagen. Cell invasion assays were performed using a Boyden Chamber invasion model. Cell invasion was inhibited 44% by YHJ-294-2 when compared with 0.5% E (ethanol). Values represent the mean ± SD from four experiments. ** $P<0.01$ (B) Kinetic assays of YHJ-294-2 inhibition activity in the upper chambers. Cell media were collected from the upper chambers 6 hours after the addition of the inhibitor. Methods are described in Figure 2. Experiments were repeated three times, yielding similar results.

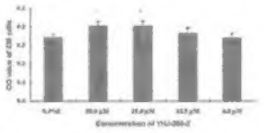


Fig. 6. The effects of YHJ-294-2 on MDA-MB-435 cell proliferation.
Proliferation assays were performed using the Cell Proliferation ELISA BrdU kit (Roche Applied Science) and manufacturer protocols. Values represent the mean ± SD from four experiments. * $P<0.05$, 0.5%ΔΣ, 0.5% ethanol

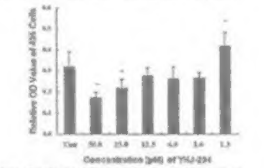


Fig. 7. The effects of YHJ-294-2 on MDA-MB-435 cell growth.
5,000 MDA-MB-435 cells/well were seeded into 24-well plate. YHJ-294-2 was then added at different concentrations every day for seven days. Cells were then fixed and cell numbers were measured as described in Fig.5. Values represent the mean ± SD from four experiments. ** $P<0.01$, Con: 0.5% ethanol

Conclusions

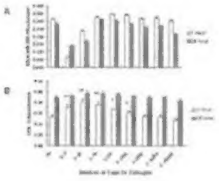
- YHJ-294-2 is a selective inhibitor for MMP-26 when compared with MMP-7 and MMP-12.
- YHJ-294-2 is stable for at least 6 hours in MDA-MB-435 cell culture systems in vitro.
- YHJ-294-2 inhibited MDA-MB-435 cell invasion through type IV collagen in vitro.
- YHJ-294-2 inhibition of MDA-MB-435 cell invasion is not secondary to its effects on cell attachment and proliferation.
- YHJ-294-2 is a potential inhibitor of breast cancer cell invasion.

THE NOVEL THREE DIMENSIONAL CELL CULTURE SYSTEM FOR HUMAN BREAST CANCER CANCER INVASION AND CELL-CELL INTERACTION*

Yongs Zhao¶, Aizhen Xiao§, Seokweo Lee§, Isa M. Hussaini¶ and Qing-Xiang Amy Sang§
§Florida State University, Tallahassee, FL 32306-4350, ¶ University of Virginia, Charlottesville VA 22908

ABSTRACT

Accumulating results showed that the expression patterns of genes and proteins are distinctly different in 3D cultures when compared with the monolayer (2D) culture commonly employed. We created a novel Polyvinyl alcohol (PVA)3D cell culture system, which consists of 3 layers. The top layer was used to grow breast cancer cells, MDA-MB-231 cells (the epithelial layer), the middle layer was saturated with ECM components (the basement membrane layer), the bottom layer was used to grow human fibroblast cells (HFL-1, the stromal layer). Then reconstituted the "sandwich" model to study breast cancer cell invasion and breast cancer cell and human fibroblast cell interaction during cancer cell progression. The results showed that both breast cancer cell (MDA-MB-231) and HFL-1 attachment varied with the different extracellular matrix components. When these two cell lines were co-cultured in monolayer, they formed tissue-like structures. When the MDA-MB-231 cell invaded into the bottom layer, which contained HFL-1 cells in the PVA 3D cell culture, the cells formed breast cancer tissue-like structure, too. Furthermore, the invaded and co-cultured MDA-MB-231 cells could be easily identified by immunohistochemically staining an epithelial-specific marker, epithelial membrane antigen. Therefore, This 3D culture system may be a useful model to study cancer cell-stromal cell interaction and cancer cells invasion. The role of the novel MMP-26 in breast cancer invasion using this 3D cell culture system is under investigation.



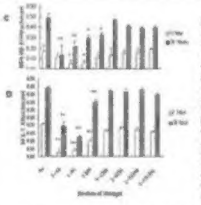


Figure 1. MDA-MB-231 cell and HFL-1 cell attachment on type IV Collagen and Matrigel. A&C : MDA-MB-231 cell attachment on Type IV collagen and Matrigel, respectively. B&D: HFL-1 cell attachment on type IV collagen and Matrigel, respectively. Statistical analysis of all samples was performed with the least significant difference (LSD) correction of ANOVA for multiple comparisons. Data represent the mean ± standard deviation (SD) from three experiments, where differences with $P<0.05$ were considered to be significant. **, $P<0.01$, * $P<0.05$.

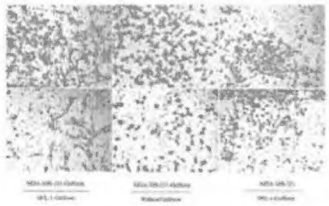


Figure 2. Morphological alteration of MDA-MB-231 cell in 3D culture system. A, MDA-MB-231-gel from co-cultured with HFL-1-gel/form, B, MDA-MB-231-gel from invasion; C, MDA-MB-231 Cell co-cultured with HFL-1-gel/form. All the pictures are 100X

Figure 3. Immunohistochemical staining of epithelial membrane antigen (EMA) in MDA-MB-231 / HFL-1 cell co-culture system. In this co-culture system, an anti-EMA antibody could be used as a marker to differentiate MDA-MB-231 cells from HFL-1 cells (Figure 3A), to the HFL-1 cells demonstrated only background staining signals (Figure 3B).

Figure 4. Immunostaining of epithelial membrane antigen (EMA) molecules in the 3D culture system. In the 3-D culture system, MDA-MB-231 cells were stained red, indicating the presence of EMA, while the underlying HFL-1 cells had only weak staining signals (Figure 4A and 4C,). Normal mouse IgG was utilized as a control, as shown in Figure 4B.

Figure 5. Expression of MMP-26 in normal and siRNA transfected MDA-MB-231 cells. Lanes 1 and 2 are cell culture media from normal MDA-MB-231 cells and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lanes 3 to 5 are cell lysates from parental MDA-MB-231 cells, MDA-MB-231 cells transfected with siRNA scramble inserts, and MMP-26 siRNA transfected MDA-MB-231 cells, respectively. Lane 6 contains purified, recombinant MMP-26 expressed in BL21 (DE3)-competent E. coli cells.

Conclusions

- Cell attachment and growth was varied with different ECM components and the concentration of ECM components.
- Epithelial membrane antigen is a reliable marker to identify MDA-MB-231 cells from HFL-1 cells in the 3D culture system.
- MMP-26 siRNA decreased 90% of MMP-26 expression in the MMP-26 siRNA transfected MDA-MB-231 cells compared with MDA-MB-231 cell and the control siRNA transfected MDA-MB-231 cells.

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